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(54) Title: METAL CHELATORS

(57) Abstract

Radionuclide chelating compounds are provided for coupling to targetting molecules such as proteins, peptides or antibodies. The resulting labelled targetting molecules may be used in diagnosis and therapy.

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METAL CHELATORS

Field of the Invention

5   This invention is in the field of diagnostic imaging, and
relates to chemical chelators useful in the
radiolabelling of agents that target tissues of
diagnostic and therapeutic interest.

Background to the Invention

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The art of diagnostic imaging exploits contrasting agents
that in binding or localizing site selectively within the
body, help to resolve the image of diagnostic interest.
^{67}Gallium-citrate, for example, has an affinity for
15   tumours and inflamed tissue and, with the aid of scanning
tomography, can reveal afflicted body regions to the
physician.   Other contrasting agents include the metal
radionuclides such as ^{99m}technetium and $^{186/188}$rhenium, and
these have been used to label targetting molecules, such
20   as proteins, peptides and antibodies that localize at
desired regions of the human body.

As targetting agents, proteins and other macromolecules
can offer the tissue specificity required for diagnostic
25   accuracy; yet labelling of these agents with metal
radionuclides is made difficult by their physical
structure.   Particularly, protein and peptide targetting
agents present numerous sites at which radionuclide
binding can occur, resulting in a product that is
30   labelled heterogeneously.   Also, despite their large
size, proteins rarely present the structural
configuration most appropriate for high affinity
radionuclide binding, i.e. a region incorporating four or
more donor atoms that form five-membered rings.   As a
35   result, radionuclides are bound typically at the more
abundant low-affinity sites, forming unstable complexes.

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To deal with the problem of background binding, Paik et
al (Nucl Med Biol 1985, 12:3) proposed a method whereby
labelling of antibody is performed in the presence of
excess DPTA (diaminetrimethylenepentaacetic acid), to
5   mask the low affinity binding sites. While the problem
of low affinity binding is alleviated by this method,
actual binding of the radionuclide, in this case
technetium, was consequently also very low. The direct
labelling of proteins having a high proportion of
10   cysteine residues also has been demonstrated (Dean et al;
WO 92/13,572). This approach exploits thiol groups of
cysteine residues as high-affinity sites for radionuclide
binding, and is necessarily limited in application to
those targetting agents having the required thiol
15   structure.

A promising alternative to the direct labelling of
targetting agents is an indirect approach, in which
targetting agent and radionuclide are coupled using a
20   chelating agent. Candidates for use as chelators are
those compounds that bind tightly to the chosen metal
radionuclide and also have a reactive functional group
for conjugation with the targetting molecule. For use in
labelling peptide and protein-based targetting agents,
25   the chelator is ideally itself peptide-based, to allow
the chelator/targetting agent to be synthesized in any
desired structural combination using peptide synthesis
techniques. For utility in diagnostic imaging, the
chelator desirably has characteristics appropriate for
30   its *in vivo* use, such as blood and renal clearance and
extravascular diffusibility.

Summary of the Invention

35   The present invention provides chelators that bind
diagnostically useful metals, and can be coupled to
targetting agents capable of localizing at body sites of

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diagnostic and therapeutic interest. The chelators of the present invention are peptide analogues designed structurally to present an N_3S configuration capable of binding oxo, dioxo and nitrido ions of radionuclides such as ^{99m}technetium and $^{186/188}$rhenium.

More particularly, and according to one aspect of the invention, there are provided metal chelators of the formula:

wherein

R_1 and R_2 together form a 5- or 6-membered heterocyclic ring which is optionally fused to a 5- or 6-membered ring, wherein either ring is optionally substituted with a conjugating group or with a conjugating group having a targetting molecule coupled thereto;

R_3 is selected from H; alkyl; and alkyl substituted by a group selected from amino, aminoacyl, carboxyl, guanidinyl, hydroxyl, thiol, phenyl, phenolyl, indolyl and imidazolyl;

R_4 is selected from hydroxyl; alkoxy; an amino acid residue; and a targetting molecule; and

T is H or a sulfur protecting group;

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In an aspect of the invention, chelators of the above formula are provided in a form having a diagnostically or therapeutically useful metal complexed therewith.

5   According to another aspect of the invention, the chelator is provided in a form coupled to a diagnostically or therapeutically useful targetting molecule. An additional aspect of the invention provides the chelators coupled to a targetting molecule and in a

10   form having a metal complexed therewith.

In another aspect of the invention, targetting molecules are provided having the general sequence: formyl-Nleu-Leu-Phe-Nleu-Tyr-Lys-Lys-Asp-X-OH wherein X is a bond or

15   an amino acid residue; the targetting molecule which may be coupled to chelators of the present invention.

Brief Description of the Figures

20   Figure 1 is a graph representing binding affinity of targetting molecules in accordance with an embodiment of the invention.

Figure 2 is a graph representing neutropenic effect of

25   targetting molecules in accordance with an embodiment of the invention.

Detailed Description of the Invention

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The invention provides chelators of diagnostically useful metals that when complexed with the metal and in a form coupled to a targetting molecule are useful for delivering the detectable metal to a body site of

35   diagnostic interest. As illustrated in the above formula, the chelators are peptidic derivatives that

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present an N_3S configuration in which the metal is
complexed.

Terms defining the variables R_1 - R_4 and T as used
hereinabove have the following meanings:

"alkyl" refers to a straight or branched C_1-C_8 chain and
includes lower C_1-C_4 alkyl;
"alkoxy" refers to straight or branched C_1-C_8 alkoxy and
        includes lower C_1-C_4 alkoxy;
"thiol" refers to a sulfhydryl group that may be
        substituted with an alkyl group to form a thioether;
"sulfur protecting group" refers to a chemical group that
        inhibits oxidation of sulfur and includes groups
        that are cleaved upon chelation of the metal.
        Suitable sulfur protecting groups include known
        alkyl, aryl, acyl, alkanoyl, aryloyl, mercaptoacyl
        and organothio groups.

In preferred embodiments of the invention, the chelators
conform to the above formula in which: R_1 and R_2 together
form a five or six membered heterocyclic ring such as
pyrrole and pyridine, or a five or six membered ring
fused to a six membered ring such as indole, quinoline
and isoquinoline; R_3 is selected from H and a hydroxy
substituted alkyl group selected from methyl and ethyl
and most preferably hydroxymethyl; R_4 is selected from
hydroxyl; alkoxy; an amino acid residue; and a targetting
molecule; and T is a hydrogen atom or the sulfur
protecting group acetamidomethyl (Acm);

In specific embodiments of the invention, the chelators
conform to the above general formula wherein T is the
sulfur protecting group acetamidomethyl (Acm); R_3 is H or
hydroxymethyl; R_1 and R_2 together form a ring selected
from pyridine, pyrrole, indole, quinoline and

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isoquinoline; and R_4 is a glycine amino acid residue or a glycine residue attached to a targetting peptide.

The substituents represented by R_1 and R_2 together with the adjacent nitrogen atom form a 5- or 6-membered heterocyclic ring which may be fused to another five or six membered ring. Five and six membered heterocyclic rings include but are not limited to pyrrole, pyrazole, imidazole, pyridine, pyrazine, pyridazine, pyrimidine and triazine. Fused rings include but are not limited to N-containing bicyclics such as quinoline, isoquinoline, indole and purine. Rings containing sulfur atoms e.g. thiazole and oxygen atoms e.g. oxazole are also encompassed by the present invention.

The heterocyclic ring formed by R_1 and R_2 may be substituted with a conjugating group that is chemically reactive allowing for coupling a targetting molecule to the chelator. In the preferred case where the targetting molecule is peptidic, the conjugating group is reactive under conditions that do not denature or otherwise adversely affect the peptide. In one embodiment of the invention, the conjugating group is reactive with a functional group of the peptidic targetting molecule such as the carboxy terminus or amino terminus. Alternatively, the conjugating group can be reactive with an ϵ-amino group of a lysine residue. Conjugating groups reactive with amino groups of targetting molecules include carboxyl and activated esters. Conjugating groups reactive with carboxyl groups of targetting molecules include amines and hydrazines.

For diagnostic and therapeutic purposes, the chelator per se may be used in combination with a detectable metal capable of forming a complex. Suitable metals include radionuclides such as technetium and rhenium in their various forms such as $^{99m}TcO^{3+}$, $^{99m}TcO_2^+$, ReO^{3+} and ReO_2^+.

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More desirably, the chelator is coupled to a targetting
molecule that serves to localize the chelated metal to a
desired location for diagnostic imaging or for therapy
ie. radiation therapy of tumours. Examples of targetting
5   molecules include, but are not limited to, steroids,
proteins, peptides, antibodies, nucleotides and
saccharides. Preferred targetting molecules include
proteins and peptides, particularly those capable of
binding with specificity to cell surface receptors
10   characteristic of a particular pathology. For instance,
disease states associated with over-expression of
particular protein receptors can be imaged by labelling
that protein or a receptor binding fragment thereof in
accordance with the present invention. Peptide-based
15   targetting molecules can be made by various known methods
or in some instances can be commercially obtained. Solid
phase synthesis employing alternating t-Boc protection
and deprotection is the preferred method of making short
peptides which can be an automated process. Recombina :
20   DNA technology is preferred for producing proteins and
long fragments thereof.

Chelators of the present invention are peptide
derivatives and are most efficiently prepared by solid-
25   phase peptide synthesis. In general solid-phase
synthesis involves the stepwise addition of amino acid
residues to a growing peptide chain that is linked to an
insoluble support or matrix, such as polystyrene. The C-
terminus residue of the chelator is first anchored to a
30   commercially available support with its amino group
protected with an N-protecting agent such as a t-
butyloxycarbonyl group (tBoc) or a
fluorenylmethoxycarbonyl (FMOC) group. The amino
protecting group is removed with suitable deprotecting
35   agents such as TFA in the case of tBOC or piperadine for
FMOC and the next amino acid residue (in N-protected
form) is added with a coupling agent such as

dicyclocarbodiimide (DCC). Upon formation of a peptide
bond the reagents are washed from the support. After
addition of the final residue, the chelator is cleaved
from the support with a suitable reagent such as
5   trifluoroacetic acid (TFA) or hydrogen fluoride (HF) and
isolated.

The present invention encompasses chelators incorporating
various heterocyclic groups containing a nitrogen atom
10 provided that it is analogous in structure to an amino
acid in that there is a carboxyl carbon, alpha carbon and
an alpha nitrogen wherein the alpha carbon and alpha
nitrogen are incorporated in a common ring. For example,
picolinic acid (pic), dipicolinic acid (dipic),
15 chelidamic acid (chel), 2-carboxypyrazine, 2-
carboxypyrimidine, 2-carboxypyrrole, 2-quinolinic acid,
1-isoquinolinic acid, 3-isoquinolinic acid and the like
will behave as a natural amino acid residue in solid
phase synthesis by forming a peptidic bond upon reaction
20 of the carboxyl group and a deprotected amino group of a
previously added residue. Variation at R_3 may be
introduced to chelators of the invention simply by
incorporation of a desired amino acid residue at the
appropriate stage of chain elongation. For example, R_3
25 may be a hydroxymethyl group by using a serine residue or
may be a hydrogen atom by using glycine. ·Any D or L,
naturally occurring or derivatized amino acid may be
used.

30 In accordance with an embodiment of the present
invention, R_4 is a targetting molecule that is
proteinaceous. Human Immunoglobulin G (HIG), a
multisubunit protein, has been directly labelled with
technetium-99m and used extensively for imaging sites of
35 inflammation, however smaller peptides are becoming the
targetting molecules of choice for their site specificity
as a result of receptor binding properties and for their

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ease of preparation. An example of peptidic targetting
molecules are Tuftsin antagonists such as Thr-Lys-Pro-
Pro-Arg and Lys-Pro-Pro-Arg. Another peptidic targetting
molecule useful for imaging inflammation is fMLP (formyl-
5 Met-Lys-Phe) and derivatives thereof such as formyl-Nleu-
Leu-Phe-Nleu-Tyr-Lys described by Fischman et al in
pending Canadian application CA 2,016,235. It is
believed that fMLP and various derivatives thereof bind
to neutrophils and are therefore useful in imaging sites
10 of inflammation.

In accordance with another aspect of the invention, the
present invention provides a peptide useful as a
targetting molecule which has the sequence formyl-Nleu-
15 Leu-Phe-Nleu-Tyr-Lys-Lys-Asp-X-OH wherein X is a bond or
an aminoacid residue. For convenient synthesis of this
peptide, X is preferrably a glycine (Gly) residue. In
vivo studies have shown this peptide coupled to a
chelator of the present invention strongly binds to
20 neutrophils while having a more favourable neutropenic
profile than native fMLP or the derivative formyl-Nleu-
Leu-Phe-Nleu-Tyr-Lys.

Synthesis of a chelator/targetting molecule conjugate
25 hereinafter referred to as a "conjugate" can be achieved
in various ways. When R_4 is a peptidic targetting
molecule, it is convenient to synthesize the conjugate *in
toto* by starting solid-phase synthesis from the C-
terminus residue of the targetting molecule and ending
30 with the heterocyclic residue (R_1, R_2) of the chelator.
Alternatively, a targetting molecule which incorporates a
lysine residue may be coupled to the chelator at R_4 by way
the ϵ-amino group of that lysine residue. In this case,
the targetting peptide is synthesized as a separate chain
35 from the chelator and is differentially protected at the
ϵ-amino group and N-terminus amino group. For example
the ϵ-amino group may be protected with 1-(4,4-dimethyl-

2,6-dioxocyclohexylidine)-ethyl (Dde) while the N-
terminus amino is FMOC protected. When the targetting
molecule synthesis is complete the ε-amino group is
deprotected with hydrazine and is available for reaction

5   with a C-terminus carboxyl group of a chelator while the
N-terminus amino group is protected.

Targetting molecules may also be coupled to chelators of
the invention by way of a conjugating group substituent

10   on the heterocyclic ring of the chelator. For example, a
chelator with an amino substituent on the heterocyclic
ring upon deprotection will be reactive with the C-
terminus carboxyl group of a peptide targetting molecule.
Such a conjugate may be synthesized as a single chain

15   starting at the C-terminus residue of the chelator and
ending with the N-terminus of the targetting molecule.
Alternatively, a peptide targetting molecule may be
coupled to the heterocyclic residue by way of its N-
terminus when the heterocyclic group has a suitable

20   conjugating group substituent such as a carboxyl group or
an activated ester. In this case, the chelator and
targetting molecule are synthesized as separate chains
and then coupled to form the desired conjugate.

25   In accordance with one aspect of the invention, chelators
incorporate a diagnostically or therapeutically useful
metal. Incorporation of the metal within the chelator
can be achieved by various methods common to the art of
coordination chemistry. When the metal is the

30   radionuclide technetium-99m, the following general
procedure may be used to form a technetium complex. A
chelator solution is formed initially by dissolving the
chelator in aqueous alcohol eg. ethanol-water 1:1. The
solution is degassed with nitrogen to remove oxygen then

35   the thiol protecting group is removed, for example with
sodium hydroxide and heat. The solution is then
neutralized with an organic acid such as acetic acid (pH

6.0-6.5). In the labelling step, sodium pertechnetate,
obtained from a Molybdemun generator, is added to the
chelator solution with an amount of stannous chloride
sufficient to reduce the technetium. The solution is
5 mixed and left to react at room temperature and then
heated on a water bath. In an alternative method,
labelling can be accomplished with the chelator solution
adjusted to pH 8. Pertechnetate may be replaced with a
solution of technetium complexed with labile ligands
10 suitable for ligand exchange reactions with the desired
chelator. Suitable ligands include tartarate, citrate,
gluconate and glucoheptonate. Stannous chloride may be
replaced with sodium dithionite as the reducing agent if
the chelating solution is alternatively adjusted to pH
15 12-13 for the labelling step. The labelled chelator may
be separated from contaminants $^{99m}TcO_4^-$ and colloidal $^{99m}TcO_2$
chromatographically, e.g. with a C-18 Sep Pak cartridge
activated with ethanol followed by dilute HCl. Eluting
with dilute HCl separates the $^{99m}TcO_4^-$, and eluting with
20 EtOH-saline 1:1 brings off the chelator while colloidal
$^{99m}TcO_2$ remains on the column. The chelators of the
invention can be coupled to a targeting molecule prior
to labelling with the radionuclide, a process referred to
as the "bifunctional chelate" method. An alternative
25 approach known as the "prelabelled ligand" method, the
chelator is first labelled with the desired metal and is
subsequently coupled to the targetting molecule. This
method is advantageous in that the targetting molecule
itself is not inadvertently labelled at low affinity
30 binding sites which may render the targetting molecule
inactive or may release the metal in vivo.

An alternative approach for labelling chelators of the
present invention involves techniques described in a co-
35 pending U.S. application 08/152,680 by Pollak et al,
filed on 16 November 1993 incorporated herein by
reference. Briefly, chelators are immobilized on a solid

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phase support in such a manner that they are released
from the support only upon formation of a complex with
the labelling metal atom. This is achieved when the
chelator is coupled to a functional group of the support
5   by one of the complexing atoms. Preferably, a complexing
sulfur atom is coupled to the support which is
functionalized with sulfur a protecting group such as
maleimide.

10   When coupled to a targetting molecule and labelled with a
diagnostically useful metal, chelators of the present
invention can be used to detect pathological conditions
by techniques common in the art. A conjugate labelled
with a radionuclide metal such as technetium may be
15   administered to a mammal by intravenous injection in a
pharmaceutically acceptable solution such as saline or
DMSO. The amount of labelled conjugate administered is
dependent upon the toxicity profile of the chosen
targetting molecule as well as the metal. Localization
20   of the metal in vivo is tracked by standard scintigraphic
techniques at appropriate time intervals subsequent to
administration.

The following examples are presented to illustrate
25   certain embodiments of the present invention.

Example 1 Preparation of Chelators and Conjugates

Chelators were synthesized using 9-
30   fluorenylmethyloxycarbonyl (FMOC) chemistry on an 2-
methoxy-4-alkoxybenzyl alcohol resin preloaded with the
protected C-terminus residue (Sasrin resin, Bachem
Biosciences Inc., Philadelphia PA) using an Applied
Biosystems 433A peptide synthesizer (Foster City, CA).
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Preparation of Chelators
a.   Chel-Gly-Cys(Acm)-Gly-OH

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        b.   DiPic-Gly-Cys(Acm)-Gly-OH
        c.   Pic-Gly-Cys(Acm)-Gly-OH

    Synthesis began from the Gly residue preloaded on the
5   resin and continued to the final Pic, DiPic or Pic
    residue by addition of one of picolinic, dipicolinic or
    cheladamic acid. The chelator-resin was dried in vacuo
    for 12 hours. Cleavage of the chelator from the resin
    involved mixing a cooled solution of 95% trifluoroacetic
10 acid (TFA) and 5% water (1ml per 100 mg of peptide-resin)
    with the peptide-resin for 1.5 to 2 hours at room
    temperature. The resin was removed by filtration and
    washed 3 times with 30 ml t-butyl methyl ether in a 50 ml
    conical polypropylene centrifuge tube forming a white
15 precipitate. The precipitate was dissolved in water with
    added acetonitrile. The precipitate was frozen in
    acetone-dry ice and lyophilized over 12 hours. The
    resulting white powder was dissolved in water, filtered
    through a 0.45 μm syringe filter (Gelman Acrodisc LC
20 PVDF) and purified by reversed-phase HPLC (Beckman System
    Gold) with a C18 column (Waters RCM 25 x 10) using 0.1%
    TFA in water as buffer A and 0.1% TFA in acetonitrile as
    buffer B. The column was equilibrated with 100:0 buffer
    A:buffer B and eluted with a linear gradient in 25 min at
25 1 ml/min to 50% buffer B. Fractions were reanalysed on
    the HPLC and pooled according to matching profiles. If
    necessary the pooled fractions were repurified using the
    same conditions. The pure fractions were frozen in
    acetone-dry ice and lyophilized over 10 hours to give a
30 white powder.

            Preparation of Conjugates
        d.   (Pic-Ser-Cys(Acm)-Gly)-Thr-Lys-Pro-Pro-Arg-OH;
        e.   (Pic-Ser-Cys(Acm)-Gly)-Lys-Pro-Pro-Arg-OH;
35   f.   2-Quinolinic acid-Ser-Cys(Acm)-Gly-Thr-Lys-Pro-
              Pro-Arg-OH;

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g.   1-Isoquinolinic acid-Ser-Cys(Acm)-Gly-Thr-Lys-
     Pro-Pro-Arg-OH;

h.   3-Isoquinolinic acid-Ser-Cys(Acm)-Gly-Thr-Lys-
     Pro-Pro-Arg-OH;

5   i.   Indole-2-carboxylic acid-Ser-Cys(Acm)-Gly-Thr-
          Lys-Pro-Pro-Arg-OH; and

     j.   Pyrrole-2-carboxylic acid-Ser-Cys(Acm)-Gly-Thr-
          Lys-Pro-Pro-Arg-OH.

10   Synthesis began from the Arg residue preloaded on the
     resin and continued to the Ser residue of the chelator
     ending with the addition of one of picolinic, 2-
     quinolinic, 1-isoquinolinic, 3-isoquinolinic, pyrrole-2-
     carboxylic and indole-2-carboxylic acid. The chelator-
15   peptide-resin was dried in vacuo 12 hours. Cleavage from
     the resin involved mixing with a solution of 10 ml
     trifluoroacetic acid (TFA), 0.5ml water, 0.5ml
     thioanisole, 0.25ml 1,2-ethanedithiol (EDT) and 0.75g
     phenol for 1.5 to 2 hours at room temperature. The resin
20   was removed by filtration and the peptide washed 3 times
     with 30 ml t-butyl methyl ether in a 50 ml conical
     polypropylene centrifuge tube forming a white
     precipitate. The precipitate was dissolved in water with
     added acetonitrile when solubility problems arose. The
25   precipitate was frozen in acetone-dry ice and lyophilized
     over 12 hours. The resulting white powder was dissolved
     in water, filtered through a 0.45 μm syringe filter
     (Gelman Acrodisc LC PVDF) and purified by reversed-phase
     HPLC (Beckman System Gold) with a C18 column (Waters RCM
30   25 x 10) using 0.1% TFA in water as buffer A and 0.1% TFA
     in acetonitrile as buffer B. The column was equilibrated
     with 100:0 buffer A:buffer B and eluted with a linear
     gradient in 25 min at 1 ml/min to 50% buffer B.
     Fractions were reanalysed on the HPLC and pooled
35   according to matching profiles. If necessary the pooled
     fractions were repurified using the same conditions. The

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pure fractions were frozen in acetone-dry ice and
lyophilized over 10 hours to give a white powder.

5   Preparation of fMLP Conjugates
k. (Pic-Gly-Cys-Gly)-εNH-Lys(-Gly-OH) -Tyr-Nleu-Phe-Leu-
Nleu-for
l. (Pic-Gly-Cys(Acm)-Gly)-εNH-Lys(-Gly-OH) -Tyr-Nleu-Phe-
Leu-Nleu-for
10 m. (Pic-Gly-Cys(Acm)-Gly)-ε-NH-Lys(-Asp-Gly-OH)-Lys-Tyr-
Nleu-Phe-Leu-Nleu-for

Targetting peptides that comprise lysine residues can be
coupled to the chelator via the Lys ε-amino group by the
15 following procedure. For compounds example 1(k), 1(l),
and 1(m). the targetting peptide was initially
synthesized from glycine to norleucine by 9-
fluorenylmethyloxycarbonyl (FMOC) chemistry using an
FMOC-glycine preloaded 2-methoxyl-4-alkoxyl-benzyl
20 alcohol resin and a 1-(4,4-dimethyl-2,6-
dioxocyclohexylidine)-ethyl (Dde) orthogonal protected
lysine with an Applied Biosystems 433A peptide
synthesizer. The fMLP peptide-resin was removed from the
synthesizer and dried 12 hours in vacuo to prepare for
25 formylation.

Formic anhydride was prepared by heating acetic anhydride
(2 equivalents) with formic acid (1 equivalent) to 50°C
for 15 minutes followed by cooling to 0°C. Formylation
30 of the fMLP peptide involved swelling the peptide-resin
in dichloromethane (DCM) (5 ml) followed by swirling with
formic anhydride (5ml) for 15 minutes. The formylated
fMLP peptide-resin was filtered, washed with DCM and
dried in vacuo 12 hours.
35

Formylated peptide-resin (50 mg/2ml) was swirled with a
2% hydrazine hydrate in N-methylpyrrolidone (NMP)

solution for 3 minutes two times then filtered and washed
with DCM and dried in vacuo 12 hours to remove the ε-
amino lysine protecting group (Dde) while leaving the N-
terminus amino group formylated.

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The chelator was added to the ε-amino lysine of the fMLP
peptide on the 433A peptide synthesizer. The chelator-
peptide-resin was dried in vacuo 12 hours. Cleavage from
the resin involved mixing a cooled solution of 95%

10 trifluoroacetic acid (TFA) and 5% water (1 ml per 100 mg
of chelator-peptide-resin) with the chelator-peptide-
resin for 1.5 to 2 hours at room temperature. The resin
was removed by filtration and washed with 1-3 ml of TFA
to obtain 6-8 ml of a clear yellow liquid. This liquid

15 was slowly dropped into 30-35 ml of tert-butyl methyl
ether in a 50 ml conical polypropylene centrifuge tube
forming a white precipitate. The precipitate was
centrifuged at 7000 rpm, 0°C for 5 minutes (Sorvall RT
6000, Dupont), decanted and washed two more times with t-

20 butyl methyl ether. Following drying under vacuum the
precipitate was dissolved in water with added
acetonitrile when solubility problems arose. The
precipitate was frozen in acetone-dry ice and lyophilized
over 10 hours. The resulting white powder was dissoved

25 in dimethylsulfoxide (20 μL) and 50:50 acetonitrile:water
solution (980 μL), filtered through a 0.45 μm syringe
filter (Gelman Acrodisc LC PVDF), and purified by
reversed-phase HPLC (Beckman System Gold) with a C18
column (Waters RCM 25 X 10) using 0.1% TFA in water as

30 buffer A and 0.1% TFA in acetonitrile as buffer B. The
column was equilibrated with 50:50 buffer A:buffer B and
eluted with a linear gradient in 25 min at 1 ml/min to
100% buffer B. Fractions were reanalysed on the HPLC and
pooled according to matching profiles. If necessary, the

35 pooled fractions were repurified using the same
conditions. The pure fractions were frozen in acetone-

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dry ice and lyophilized over 12 hours to give a white
powder.

Example 2 Labelling Chelators with ^{99m}Tc

5

The chelators and conjugates of example 1 (1mg) were
dissolved in 200μL EtOH-water (1:1) in a tube.   100-200μL
sodium pertechnetate (200-600 MBa, 5-15 mCi), 100 μL
phosphate buffer (0.25 M, pH 7.4), and 200 μL of a
10   solution containing 50μg stannous chloride dihydrate and
40mg sodium tartrate were added to the tube and capped
tightly and placed in a boiling water bath for 10
minutes.   In order to achieve adequate separation of the
chelators, the solution was then loaded on a C-18 Sep-Pak
15   column activated by washing sequentially with 5ml
methanol, 10ml water and 5ml dilute (1mM) HCl to remove
TcO_4^-.   Subsequent elution with 2ml EtOH-saline (1:1)
removed the chelator while TcO_2 remained on the column.
The extent of complexation of ^{99m}Tc with chelators was
20   measured by radioactivity of the eluted fractions.

Example	Labelling Yield (% total radioactivity)
1(a)	7
1(c)	92
1(d)	93
1(e)	92
1(k)	29
1(l)	63
1(m)	78

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Conjugates 1(f) - 1(j), were reconstituted (200µL, 1mg/mL saline) and then injected into 3mL vacutainers with 100µL pertechnetate (10mCi) and 100µL stannous gluconate (50 µg stannous chloride and 1 mg sodium gluconate). The tubes

5   were placed in boiling water bath for 12 minutes and then filtered through a Whatman PVDF syringe filter to collect the labelled conjugate solutions which were further diluted with saline to prepare injectable solutions (2Mbq/mL). The conjugates were isolated by HPLC

10   (Beckman) from a (20µL) sample (before dilution) to determine the labelling yield by measuring radioactivity.

example	Labelling Yield (%)
1(f)	94.4
1(g)	96.8
1(h)	94
1(i)	96.3
1(j)	98.4

15

20

Example 3   In vivo imaging and biodistribution of chelators and conjugates

For chelators and conjugates of example 1, rat

25   inflammation studies were performed as follows. 2 male Sprague-Dawley rats (Charles River, 250-300g) were injected intramuscularly with 5mg zymosan, a yeast cell wall preparation (20mg for conjugates 1(f) - 1(i)) or a virulent E.coli (ATCC 25922, 0.1ml of 1.0X10^9

30   organisms/ml) suspension into their right hindlegs 24 hours before imaging. Focal inflammation in the leg was visually detectable after 1 day. 1mg (ca. 0.7 µMol) of the chelator was dissolved in 50 µL of dimethylsulfoxide and added to an ethanol-water mixture (1:1, 200 µL). An

35   aliquot of Tc-99m tartarate (ca. 400 MBq) was added and

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transchelation allowed to proceed for 20 min. at 100°C.
The Tc-99m chelate was purified by elution through a Sep
Pak cartridge.   The purified tracer solution was further
diluted with saline to prepare an injectable (200 µL)
5   containing about 100 µCi (3.7 MBq) of activity.

The rats were anaesthetized with sodium pentobarbital (40
to 50 mg/kg), and the labelled chelator/conjugate
solution (200µL) was injected intravenously via the tail
10   vein.   Serial whole-body scintigrams were acquired for
the first 5 minutes. Subsequently, further images were
obtained at 30, 60, and 120 minutes.   The rats were then
killed with anaesthesia and samples of organs, urine,
blood, inflamed muscle (right leg) and non-inflamed
15   muscle (left leg) were weighed and counted in either a
well-type gamma counter or in a gamma dose calibrator.
The dose calculations were made based on assumption that
the blood volume constituted 6.5% of body weight.
Results are averages for two rats and are corrected for
20   the residual dose in the tail.

In vivo Distribution

example	blood	liver	kidney	urine	GI tract	time (min)	Inflam muscle	Uninfl muscle	Inflam agent	Infl: Uninfl	time (min)
			(% per organ)					(% per g)			
1(a)	1.016	0.953	4.359	33.950	52.619	88			none		
1(c)	0.187		21.700				0.077	0.019	E.coli	4.24	
1(d)	6.370	3.050	4.170	44.710	5.080	30	0.180	0.050	E.coli	3.62	30
1(e)	3.520			53.000		30	0.125	0.059	E.coli	2.1	
1(f)	5.360	4.731	4.037	51.372	4.416	35	0.095	0.025	zymosan	4.8	35
1(g)	5.154	3.219	6.656	43.783	7.164	35	0.132	0.030	zymosan	4.4	35
1(h)	5.187	3.155	3.077	58.460	4.117	35	0.101	0.030	zymosan	3.7	35
1(i)	8.674	4.989	2.330	40.274	39.179	35	0.177	0.035	zymosan	5.1	35
1(k)	3.036	35.26	18.82	8.400	41.285	90			zymosan		
1(l)	3.436	20.866	11.470	14.050	25.777	109	0.143	0.041	E.coli	3.17	
1(m)							0.104	0.052	E.coli	3.52	45
IgG	48					120			E.coli	2.07	120

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Example 4 Neutrophil Binding Assay of fMLP and fMLP
Derivative Conjugates of Example 1(l) and 1(m)

Rat peripheral neutrophils were prepared for binding
5   assay as follows: blood was obtained by cardiac puncture
and anticoagulated with acid- citrate dextrose (ACD)
(10%). Red blood cells were removed by sedimentation on
hydroxyethyl cellulose (1.1%) for 30 min at room
temperature and leukocyte-rich supernatant layered onto
10   65% percol. Centrifugation at 400g for 30 min resulted
in a distinct band of mononuclear cells (lymphocytes and
monocytes) which was discarded, the neutrophil rich
pellet was resuspended and remaining red blood cells
lysed by hypotonic shock using cold water. The remaining
15   neutrophils were resuspended in Hanks Buffered Salt
Solution (HBSS) to the desired concentration. Final
neutrophil preparation consisted of cells pooled from up
to 10 animals, >90% neutrophils and >95% viable by Trypan
Blue exclusion.
20

Binding affinity of the fMLP peptide and conjugates 1(l)
and 1(m) was assessed by competing off a constant
concentration of tritiated fMLP of known affinity for
neutrophil receptors. 10^6 neutrophils were added to
25   polypropylene plates containing 15nM tritiated fMLP and
varying concentrations of unlabelled test peptide and
conjugates in a final volume of 150μL HBSS. The plate
was incubated for 1 hour at room temperature after which
cells were harvested by filtration onto glass fibre
30   filter mats (Skatron receptor binding filtermat) using a
Skatron cell harvester with 12 well head. Harvested
cells were washed with ice-cold saline and air dried.
Filters were then placed in 6ml scintillation vials, 5ml
of scintillation fluid added (Ecolume) and vials counted
35   using a liquid scintillation counter. Binding affinity
of the fMLP peptide and conjugates 1(l) and 1(m) is
illustrated in figure 1 and expressed as % maximal

tritiated fMLP binding vs. peptide/conjugate
concentration. % maximal tritiated fMLP
binding=(specific binding ÷ maximum binding) X 100%.
Specific binding was the total binding less non-specific
5 binding which was the amount of residual radioactivity
bound in the presence of 10μM unlabelled fMLP. Both 1(l)
and 1(m) had greater binding affinity for neutrophil
receptors than native fMLP.

10 <u>Example 5</u> Neutropenia Assay of fMLP, fMLP Derivative
formyl-Nleu-Leu-Phe-Nleu-Tyr-Lys and Conjugate 1(m)

The effect of fMLP, fMLP derivative formyl-Nleu-Leu-Phe-
Nleu-Tyr-Lys and conjugate of example 1(m) on circulating
15 neutrophil number was assessed using the rat transient
neutropenia model. Rats were anaesthetized with 250μL
somnitol (16mg/rat) and injected via the tail vein at T=0
with the test peptides. At a range of time points after
injection (0, 2, 5, 10, 30 min) a 2ml blood sample was
20 taken by cardiac puncture (anticoagulated with 10% ACD).
3 animal were used per time point. For each sample the
total white blood cells/ml and % neutrophils was
determined, the number of neutrophils/ml in each sample
being calculated. Within each experiment the number
25 neutrophils/ml after saline injection at all time points
was meaned to give a saline control against which the
peptides could be compared. The number neutrophils/ml
after peptide injection was expressed as a % of the
saline control within each experiment.
30

Referring to figure 2, injections of 5 and 10 nmoles of
fMLP produced a dose-dependent transient neutropenia,
with a maximal effect occurring 2 min after peptide
injection (15 and 9% of control respectively) returning
35 to 93 and 75% of control values by 30 min after
injection. 5nmoles of formyl-Nleu-Leu-Phe-Nleu-Tyr-Lys
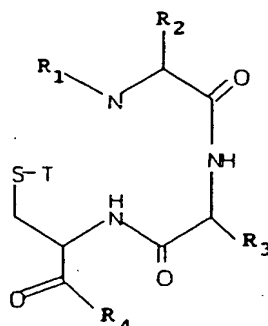produced a smaller maximal reduction in circulating

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neutrophils (45% of control) while 1(m) produced only a small transient drop in circulating neutrophils (80% of control) at 5nmoles.

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WE CLAIM:

1. A compound of the general formula:

wherein

    R_1 and R_2

         together form a 5- or 6-membered heterocyclic
         ring which is optionally fused to a 5- or 6-
         membered ring, wherein either ring is
         optionally substituted with a conjugating group
         or with a conjugating group having a targetting
         molecule coupled thereto;

    R_3 is selected from H; alkyl; and alkyl substituted
         by a group selected from amino, aminoacyl,
         carboxyl, guanidinyl, hydroxyl, thiol, phenyl,
         phenolyl, indolyl and imidazolyl;

    R_4 is selected from hydroxyl; alkoxy; an amino acid
         residue; and a targetting molecule; and

    T is H or a sulfur protecting group;

2. A compound according to claim 1, wherein the ring
formed by R_1 and R_2 is a five or six membered heterocyclic
ring optionally fused to a benzene ring.

3. A compound according to claim 1, wherein R_1 and R_2
together form a ring selected from pyridine, quinoline,
isoquinoline, pyrrole and indole.

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4.   A compound according to claim 1, wherein R_1 and R_2 together form a ring selected from 6-carboxypyridine and 4-hydroxy-6-carboxypyridine.

5   5.   A compound according to claim 1 wherein R_3 is selected from H and hydroxymethyl.

6.   A compound according to claim 1, wherein R_4 is selected from -Gly-OH and -Gly-targetting molecule.

10

7.   A compound according to claim 6, wherein the targetting molecule is a peptide.

8.   A compound according to claim 7, wherein the peptide
15   has a sequence selected from -NH-Lys-Pro-Pro-Arg-OH; and -NH-Thr-Lys-Pro-Pro-Arg-OH.

9.   A compound according to claim 7, wherein the Gly forms an amide linkage with an ϵ-amino Lys residue of the
20   peptide selected from:
    -ϵ-amino Lys(-Gly-OH)-Tyr-Nleu-Phe-Leu-Nleu-formyl; and
    -ϵ-amino Lys(-Asp-Gly-OH)-Lys-Tyr-Nleu-Phe-Leu-Nleu-formyl.

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10.   A compound according to claim 1, wherein R_1 and R_2 together form a pyridine ring; R_3 is H; T is Acm; and R_4 is selected from:
    -Gly-OH; and
30   -Gly-ϵ-amino Lys(-Gly-OH)-Tyr-Nleu-Phe-Leu-Nleu-formyl.

11.   A compound according to claim 1, wherein R_1 and R_2 together form a pyridine ring; R_3 is hydroxymethyl; T is
35   Acm; and R_4 is selected from:
    -Gly-OH;
    -Gly-Lys-Pro-Pro-Arg-OH;

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-Gly-Thr-Lys-Pro-Pro-Arg-OH;

-Gly-ε-amino Lys(-Gly-OH)-Tyr-Nleu-Phe-Leu-Nleu-formyl; and

-Gly-ε-amino Lys(-Asp-Gly-OH)-Lys-Tyr-Nleu-Phe-Leu-
5   Nleu-formyl.

12.   A compound according to claim 1, selected from:
      2-Quinolinic acid-Ser-Cys(Acm)-Gly-Thr-Lys-Pro-Pro-
Arg-OH;

10   1-Isoquinolinic acid-Ser-Cys(Acm)-Gly-Thr-Lys-Pro-
Pro-Arg-OH;

          3-Isoquinolinic acid-Ser-Cys(Acm)-Gly-Thr-Lys-Pro-
Pro-Arg-OH;

          Pyrrole-2-carboxylic acid-Ser-Cys(Acm)-Gly-Thr-Lys-
15   Pro-Pro-Arg-OH; and

          Indole-2-carboxylic acid-Ser-Cys(Acm)-Gly-Thr-Lys-
Pro-Pro-Arg-OH.

13.   A compound according to claim 1, wherein R_1 and R_2
20   together form a ring selected from a 6-carboxypyridine
ring and a 6-carboxy-4-hydroxypyridine ring; R_3 is
hydroxymethyl; T is Acm; and R_4 is -Gly-OH.

14.   A compound according to claim 1, in a form complexed
25   with a metal or an oxide or nitride thereof.

15.   A compound according to claim 10, wherein the metal
is ^{99m}Tc.

30 16.   A compound according to claim 11, in a form
complexed with ^{99m}Tc.

17.   A compound according to claim 12, in a form
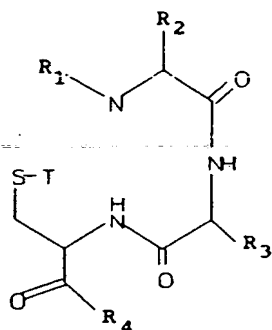complexed with ^{99m}Tc.

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18. A method of imaging for sites of in vivo localization of a targetting molecule comprising the steps:

    1) administering a diagnostically effective amount of a compound according to claim 15, comprising said targetting molecule; and

    2) detecting localization of the compound.

19. A compound of the general formula:

wherein:

    R_1 and R_2 together form a 5- or 6-membered heterocyclic ring which is optionally fused to a 5- or 6-membered ring, wherein either ring is optionally substituted with a conjugating group or with a conjugating group having a targetting molecule coupled thereto;

    R_3 is selected from H; alkyl; and alkyl substituted by a group selected from amino, aminoacyl, carboxyl, guanidinyl, hydroxyl, thiol, phenyl, phenolyl, indolyl and imidazolyl;

    R_4 is selected from hydroxyl; alkoxy; an amino acid residue; and a targetting molecule; and

    T is H or a sulfur protecting group;

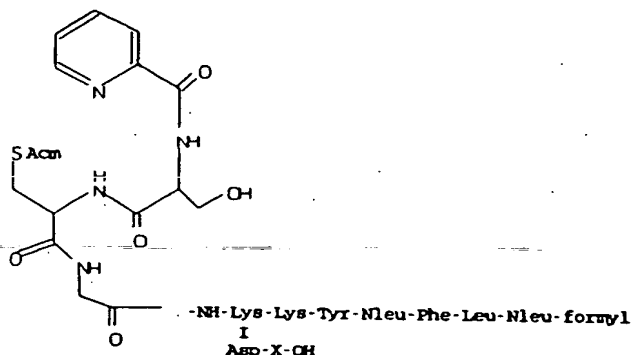   wherein the targetting molecule is a peptide having a sequence:

    formyl-Nleu-Leu-Phe-Nleu-Tyr-Lys-Lys-Asp-X-OH

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and X is a bond or an amino acid residue.

20. A compound according to claim 19, wherein X is
-Gly-.

21. A compound according to claim 19, having the
formula:

wherein X is a bond or an amino acid residue.

22. A compound according to claim 21, wherein X is
-Gly-.

23. A peptide having the general sequence:

formyl-Nleu-Leu-Phe-Nleu-Tyr-Lys-Lys-Asp-X-OH

wherein X is a bond or an amino acid residue.

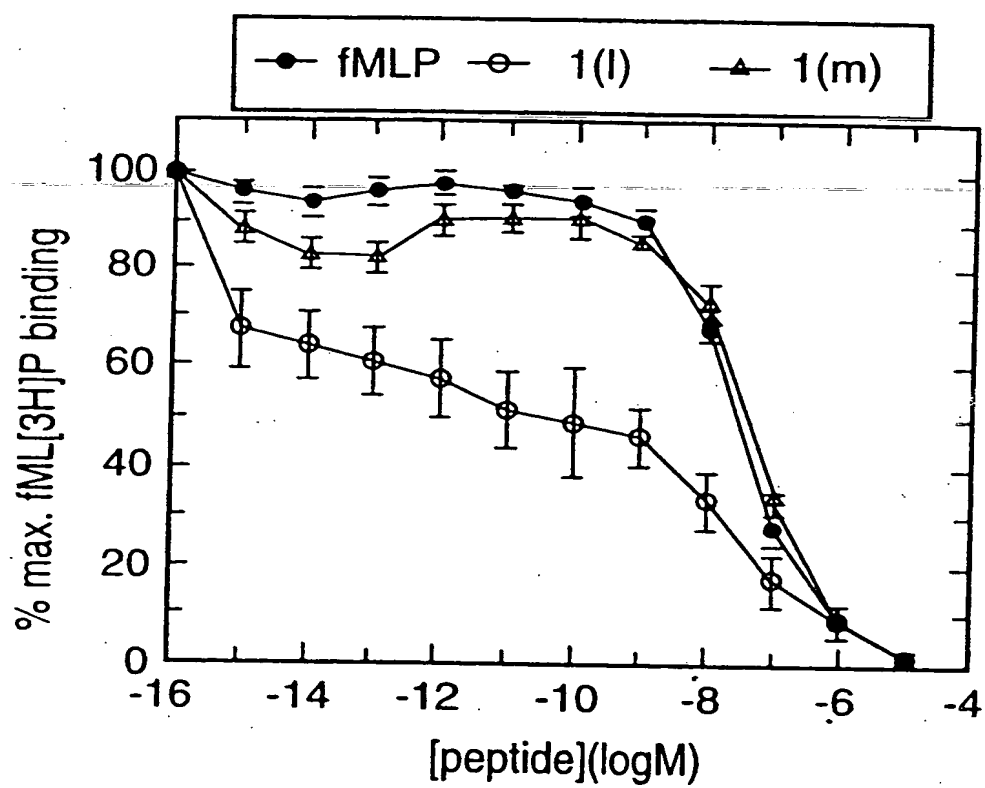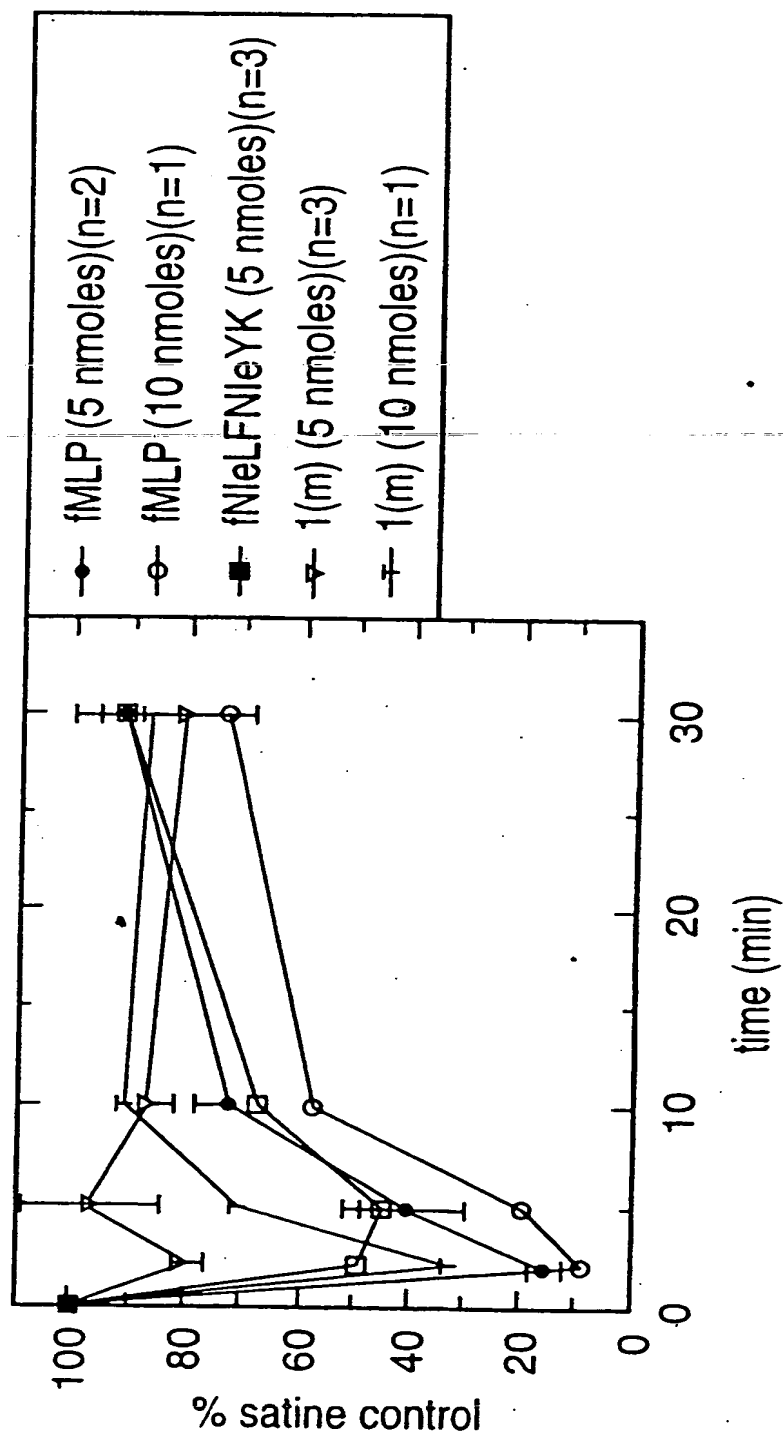24. A peptide according to claim 23, wherein X is -Gly-.

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F I G.1

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FIG.2.

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K5/097 C07K5/06 A61K51/08 A61K51/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,93 23085 (DIATECH,INC.) 25 November 1993	1-7,14
Y	see page 9, line 16 - page 10, line 6 see page 17, line 18 - line 22 see page 49; claim 59	1-7,9-24
X	INT.J.PEPTIDE PROTEIN RES., vol. 5, 1973 pages 91-98, AKHTAR A. ET AL. 'SYNTHESIS OF A CHELATED CORE RELATED TO RUBREDOXIN' see example X	1-3
X	WO,A,93 22338 (RIJKSUNIVERSITEIT LEIDEN) 11 November 1993 *peptid PLCDLLIRC* see claim 5	1-3

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[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
29 March 1995	11-05-1995

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Deffner, C-A

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP,A,0 398 143 (THE GENERAL HOSPITAL CORPORATION) 22 November 1990 see the whole document ---	1-7,9-24
A	US,A,4 687 840 (PKT. PANG ET AL.) 18 August 1987 -----	

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		CA-A-	2136330	25-11-93
		EP-A-	0641222	08-03-95
WO-A-9322338	11-11-93	AU-B-	4358693	29-11-93
		CA-A-	2112798	11-11-93
		EP-A-	0593754	27-04-94
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		CA-A-	2016235	09-11-90
		JP-T-	4505761	08-10-92
		WO-A-	9013317	15-11-90
US-A-4687840	18-08-87	NONE		